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Note

The isolation and characterization of hyaluronic acid from *Pasteurella multocida*

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The pathogenicity and destructiveness of *Pasteurella multocida* in various disease conditions of domestic animals and fowl has been of economic concern for many years and has led to extensive studies aimed at the elucidation of substances related to this pathogenicity[1–5] Four types of *P multocida* have been designated by Carter[1]; of these only one, type A, has been reported to possess capsules which possibly contain hyaluronic acid Evidence for this suggestion was based primarily on the observation that staphylococcal hyaluronidase removed capsules from growing cells[1], an action cited by Carter and Annau[2] to be similar to that occurring with the capsules of streptococcal cells

Soluble capsular products were isolated which also were affected by hyaluronidase, as indicated by turbidity reduction with acid albumen[6] It was noted that, after hydrolysis with acid, the soluble material produced reducing substances However, analytical data establishing the presence of hyaluronic acid were not given, and the relation of the soluble capsular products to hyaluronic acid from other sources was uncertain.

In the present study, a highly purified, electrophoretically homogeneous polysaccharide preparation was isolated from *P. multocida* (type A, Strain X-73, ATCC 11039), a highly virulent strain associated with fowl cholera[3] Chemical characterisation of this product indicated its identity with hyaluronic acid of streptococcal origin (Group A, type 18, Strain A III)[7] *P multocida* cells were grown in Adams–Roe medium[8], with or without the extra addition of sodium hydrogen carbonate Seed cultures were obtained by incubating (24 h, 37°) lyophilized cells on plates containing dextrose–starch agar (Difco) Subsequently isolated colonies were grown in tubes containing Adams–Roe media[7], except that thioglycollate and sodium

hydrogen carbonate were omitted After 24 h the content of a single tube was added to 3 liters of Adams–Roe medium, and this was incubated under the same conditions. After addition of 9 ml of formalin (37%), the cells were recovered by centrifugation (RC-2 Servall) at 9000 r p m for 1 h The cells were suspended in 0 85% sodium chloride containing 0 3% formalin, and the suspension was kept in a refrigerator. The dry weight of cells produced from 3 liters of medium was 2 5 g for cultures containing sodium hydrogen carbonate and 1 6 g for those grown without this addition

Isolation of the polysaccharide was accomplished by the following method The cells from a 3-liter culture were suspended in 50 ml of a buffer solution (pH 5 0) containing 0.15M sodium chloride and 0 10M sodium acetate Crystalline papain (20 mg), Versene (30 mg), and cysteine hydrochloride (10 mg) were added, and the mixture was incubated with stirring for 18 h at 60–65° Centrifugation at 10,000 *g* for 30 min provided a clear, yellow supernatant solution The sediment was found to contain an additional amount of hyaluronic acid which was recovered as indicated later

The supernatant solution was concentrated to 10 ml *in vacuo*, and 2 volumes of ethanol were added After separation, this crude preparation weighed approximately 200 mg No significant differences between the yields of polysaccharide obtained from cells grown with or without sodium hydrogen carbonate were noted

The crude material was heavily contaminated with protein (Table I) and with nucleic acid, as indicated by the u.v. spectrum, and it was fractionated by precipitation with cetylpyridinium chloride in 0 1M sodium chloride[9]. Final purification was performed by precipitation of the mucopolysaccharide with an alkaline copper solution, as described for the isolation of dermatan sulfate[10]. The yield of purified material obtained after this step was 60–70 mg An additional 20–30 mg of copper-precipitated polysaccharide was recovered after alkaline extraction of the cellular sediment. The total yield of hyaluronic acid was up to 4–5% of the dry weight of *P multocida* On some occasions, much smaller yields than those reported here were obtained, and in such instances varying amounts of polysaccharides containing neutral sugars were isolated, in addition to hyaluronic acid No hyaluronic acid was detected in extracts from *P. multocida*, type B, Strain 1404

The use of Dowex-1 for fractionation of the crude polysaccharide gave a relatively pure product, but it was difficult to obtain reasonable yields, and only one-half or less was generally recovered after elution under the usual conditions The data in Table I illustrate the similarity in composition between the polysaccharide from *P. multocida* and the hyaluronic acid from a streptococcal source[11]. The fraction obtained as the copper complex was not contaminated with compounds containing 2-amino-2-deoxy-D-galactose. Furthermore, u v. absorption spectra showed that only the copper-precipitated sample was free of nucleic acid The difficulty experienced in obtaining reasonably pure hyaluronic acid from *P multocida* contrasts with the relative ease in isolating this polysaccharide from streptococcal extracts, although the purity of preparations obtained as the copper complexes are comparable, as judged by their compositions

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TABLE I

COMPOSITION[a] OF HYALURONIC ACID PREPARATIONS FROM *Pasteurella Multocida* AND GROUP A STREPTOCOCCUS

Source and conditions of preparation	*N*, %	*Uronic acid*, %	*2-Amino-2 deoxy-D-glucose*, %	$[\alpha]_D^{25}$ (*in water*), *degrees*	*Amino Acid Autoanalyses*[b]								
					GalN	*Asp*	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Total of others amino acids*
P Multocida													
Ethanol	8 6	11 9	10 6		0 052	0 109	0 060	0 069	0 107	0 073	0 195	0 092	0 282
Cetylpyridinium chloride	3 9	37 3	28 3										
Alkaline-copper reagent	2 8	40 8	30 6	−63	0 001	0 006	0 004	0 009	0 007	traces	0 011	0 007	0 026
Streptococcus Alkaline-copper reagent	3 2	44 4	35 1	−70	0 001	0 006	0.007	0 010	0 013	traces	0.007	0 008	0 010

[a]Analytical methods described in Ref 17, the results have been corrected for moisture content, and the hexosamine values were taken from the amino acid autoanalysis data and were not corrected for destruction occurring under the conditions used [b]Expressed as ratios to 2-amino-2 deoxyglucose

The identity of the *P multocida* polysaccharide with hyaluronic acid was confirmed by the similarity of its electrophoretic mobility[12] with that of streptococcal hyaluronic acid (Fig 1) and by the disappearance of turbidity with acid albumen[6], as well as by the formation of products having an absorption maximum at 232 nm[13] after incubation with streptococcal hyaluronidase An ion-exchange chromatographic method developed for the detection of uronic acids[14] showed the presence of glucuronic acid only The purified hyaluronic acid emerged with the void volume after gel filtration through Sephadex G-200, as described by Wasteson[15], resembling in this respect the hyaluronic acid from other sources Treatment with alkali, therefore, appears not to have greatly affected the molecular size of the hyaluronic acid, in agreement with the findings of Swann[16] that alkali treatment of hyaluronic acid from rooster combs produced insignificant changes in molecular size

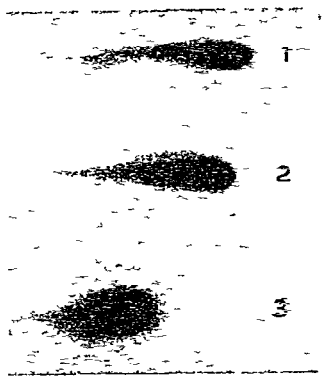

Fig 1 Electrophoretic mobilities of hyaluronic acid preparations The polysaccharides were applied in a 0 5% solution to Sepraphore III cellulose acetate strips, run for 45 min at 170 V in a pyridine–formate buffer, pH 3 0, as described by Mathews[12], and stained with Acridine Orange, (1) purified hyaluronic acid from *P multocida*, (2) streptococcal hyaluronic acid (3) purified hyaluronic acid from vitreous humor

In contrast to the similarity of the properties of hyaluronic acids obtained from Pasteurella and Streptococcus, the stability of the capsules of these bacteria appears to differ greatly Thus, hyaluronic acid is readily released from streptococcal cells into the culture medium, whereas the capsules of Pasteurella are removed with difficulty and, even after digestion with papain for 18 h, significant amounts of capsular material are attached to the cells.

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